Application Note

Cell Analysis

Agilent

Trusted Answers

Real-Time Cell-by-Cell Analysis with Agilent xCELLigence RTCA eSight

For dynamic tracking and classification of live-cell heterogeneity assays

Authors

Tian Wang, Ryan Raver, Yunfei Pu, Grace Yang, Peifang Ye, Xiaoyu Zhang, and Nancy Li

Agilent Technologies, Inc.

Abstract

Heterogeneity is mirrored by the diversity of pharmacological response at the cellular level, where even seemingly identical cells may respond differently and at varying times to drug treatments. Thus, identifying and quantifying the status and function of cell subsets is fundamental to all cell research, especially for tumor immunity, as it can provide valuable additional biological insight beyond that which whole population measures may deliver. Current methods for obtaining cell subset quantitative measurements rely on established biomarkers. Flow cytometry is a widely adopted method for discriminating and quantifying subpopulations based on cellular phenotype. However, increased imaging resolution and optimized data analysis algorithms are making long-duration live-cell imaging systems with cell-by-cell analysis competitive at the single-cell level. In this application note, we demonstrate the utility of a cell-by-cell analysis module that uses brightfield imaging to enable label-free cell segmentation and classification based on area, eccentricity, or fluorescence intensity, allowing for real-time tracking and quantification of dynamic changes in cell subpopulations within a mixed culture. Using the Agilent xCELLigence RTCA eSight Cell-by-Cell Analysis module, we show that (1) the label-free brightfield cell counts closely match fluorescent nuclear counts in both adherent A549 and suspension K562 cell lines. (2) The HCC1954 cell line is human epidermal growth factor receptor 2 (HER2) positive and the A549 cell is HER2 negative—consistent with the literature. (3) The cell counts at different stages of cell death can be kinetically monitored in a camptothecin (CPT)-mediated HT-1080 cytotoxicity assay. (4) There is a 5-Fluorouracil (5-FU) dose-dependent effect on cell cycle progression of HT-1080 cells. Taken together, we conclude that label-free brightfield segmentation of the xCELLigence RTCA eSight system is robust and that cell-by-cell analysis is a powerful tool for kinetic analysis of heterogeneous cell subsets.

Introduction

Cellular heterogeneity is a prevalent biological phenomenon, characterized by variability in genes, epigenetics, and phenotypes among morphologically indistinguishable cells.[1] This diversity plays a pivotal role in tissue biology and disease progression. For instance, tumor heterogeneity gives rise to disparities in growth rate, invasiveness, drug sensitivity, and disease prognosis, among different tumors and within the same tumor at distinct sites.[2,3] The ability to analyze at a cell-by-cell level enables the characterization of cellular subsets and the ability to monitor their individual response to various stimuli, which is crucial in modern molecular biology. This is particularly true in tumor immunity studies, including the classification and quantification of lymphoid T and B cell populations in mixed peripheral blood mononuclear cell (PBMC) cultures, the differentiation of T cells of different immune subtypes, and the differentiation of subpopulations of cells potentially benefiting from targeted therapies in tumors.

Flow cytometry enables rapid, quantitative, and multiparametric fluorescence analysis of heterogeneous cell populations at the individual cell level (that is, single-cell analysis). This process employs antibodies to target cellular markers—functional membrane proteins expressed by the cells involved in cellular communication, adhesion, or metabolism. By leveraging these cellular markers, flow cytometry proves to be an efficacious approach to discern and categorize subpopulations within complex cell populations.[4]

In contrast to endpoint assays that require cell fixation and staining at a specific time point, real-time live-cell methods provide a more representative view of dynamic cellular responses over time under normal physiological conditions. Live-cell imaging systems combine automated multicolor fluorescence with quantitative data analysis to measure and monitor phenotypic changes. This enables the assessment of multiple molecular parameters and characteristics, such as cellular activity, protein expression, receptor internalization, and cell cycle in individual cells within the cell culture.

The Agilent xCELLigence RTCA eSight system is a real-time live-cell imaging and automated analysis system that operates in a standard CO_2 cell incubator, and aside from inoculating and treating cells, requires no manual cell operations. Here, we introduce a label-free Cell-by-Cell Analysis module for the RTCA eSight system that uses an image processing algorithm to perform brightfield segmentation and subsequent cell-by-cell analysis of cell count, area, eccentricity, or fluorescence intensity. The label-free cell-by-cell analysis capability allows for real-time tracking of subpopulation-state temporal dynamics in a mixture population.

In summary, this application note demonstrates the ability of the RTCA eSight system to perform label-free counting of adherent (A549) or nonadherent (K562) cells, to determine human epidermal growth factor receptor 2 (HER2) expression using anti-HER2 BV421 antibodies, and to quantify different subpopulations with healthy status after camptothecin (CPT)-induced cytotoxicity in HT-1080 cells. In addition, the RTCA eSight system performed cell cycle analysis of HT-1080 cells expressing fluorescent ubiquitination-based cell cycle indicator (FUCCI) after being treated with 5-Fluorouracil (5-FU), a G1 arresting anticancer drug, demonstrating a dose-dependent effect on cell cycle progression.

Assay principle

The RTCA eSight instrument interrogates cell state and behavior in real time using a combination of cellular impedance and live-cell imaging. It is capable of label-free biosensor monitoring and kinetic imaging of the same live cell population individually or simultaneously. The instrument is placed in a 37 °C/5% CO_2 incubator, enabling long-term studies that can span weeks. The integration of impedance biosensors with brightfield and three-color fluorescence imaging adds dimension to the cell analysis data collected, rendering it suitable to conduct investigations on cellular activity, immune cytotoxicity, monoclonal assays, scratch experiments, and immune cell killing of 3D cell culture. Prior to Agilent RTCA eSight software version 1.2.1, data analysis was based on the overall whole cell population within the imaging field of view. A homogeneous mixture of cell populations can be seeded evenly in various wells to evaluate the differences in stimulation response between wells. However, variations in response resulting from heterogeneity within the cell population are concealed by population-level responses and thus disregarded. As such, it was previously not possible to differentiate and pinpoint the variable effects of a treatment on a subset of cells from the entire cell population.

To address this gap, we developed the Cell-by-Cell Analysis module to accurately analyze and quantify the subpopulation characteristics over time utilizing cell surface markers of cell health and/or functional properties (Figure 1). The introduction of cell-by-cell analysis to the RTCA eSight system is a valuable enhancement that enables label-free single-cell segmentation based on brightfield imaging. Using this functionality, researchers can easily and automatically count adherent or nonadherent cells free of labels (Figures 2 and 3). Furthermore, users can identify and quantify cell subsets based on their morphological features (area and

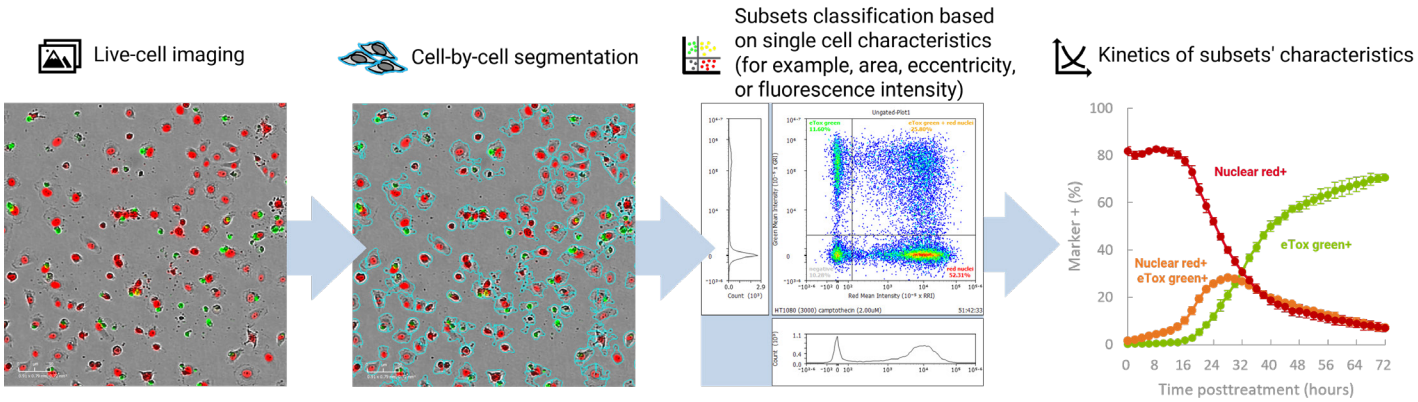

Figure 1. Agilent xCELLigence RTCA eSight cell-by-cell analysis workflow using the assay data from CPT-induced cytotoxicity of HT-1080 cells as an example.

eccentricity) and internal fluorescence characteristics (intensity in red, green, or blue fluorescence) that reflect cell state and characteristics. The distribution of cells for each feature at every time point is viewed using bivariate plots and histograms (Figures 5B). Cell subsets are indicated in the cell image by a simple colored mask (Figure 5A), and each cell in the image is localized in the bivariate plot. Plotting the characteristics of each subset over time enables the kinetic study of cell subsets (Figures 5C, 5D, and 5E).

Experimental

Cell maintenance and assays were conducted at 37 °C/ 5% CO_2. Cell lines and their growth media are shown in Table 1. FBS and sodium bicarbonate 7.5% solution were purchased from Gibco (part numbers 10099-141 and 25080094, respectively) and penicillin/streptomycin (pen/strep) was purchased from HyClone (part number SV30010).

Table 1. Cell lines used and their respective growth media.

Cell Lines	Base Medium	Medium Supplements
K562	IMDM (Gibco, p/n A10489-01)	10% FBS + 1% pen/strep
A549	Ham's F-12K (Gibco, p/n 21127-022)	10% FBS + 1% pen/strep
HCC1954	RPMI 1640 (Gibco, p/n 11875-093)	10% FBS + 1% pen/strep
HT-1080	EMEM (ATCC, p/n 30-2003)	10% FBS + 1% pen/strep

Accurate cell counting based on brightfield segmentation

K562 Green cells, which express a nuclear-localized green fluorescent protein (GFP), were produced by transducing the parental cells with Agilent eLenti Green reagent (part number 8711010). K562 cells at the logarithmic growth stage were collected and seeded on the plate at 80K, 40K, 20K, 10K, 5K, 2.5K, 1.25K, or 0.625K cells/well (three replicates for each group). In the same way, A549 Blue cells, produced by transducing parental cells with Agilent eLenti Blue reagent (part number 8711012), were deposited into a 96-well plate at a series of cell densities (4K, 2K, 1K, 0.5K, 0.25K, or 0.125K cells/well). Cell proliferation was then monitored continuously for three days.

HER2 expression characterization by anti-HER2 BV421 antibody labeling

A549 cells and HCC1954 cells were seeded into a 96-well plate at a density of 5K cells/well. At 24 hours post-cell-seeding, 0.03 µg IgG isotype antibody (Biolegend, part number 400157) or anti-HER2 BV421 antibody (Biolegend, part number 324420) was added to each well separately. HER2 expression was detected by RTCA eSight afterward.

Camptothecin-induced cytotoxicity on HT-1080 cells

HT-1080 Red cells, produced by transducing parental cells with Agilent eLenti Red reagent (part number 8711011), were collected and seeded into a 96-well plate at 2.5K cells/well. At 24 hours post-cell-seeding, the plate was removed from the RTCA eSight and 50 µL of medium containing both CPT (at the final concentrations of 2 µM, 0.5 µM, 0.13 µM, 31.3 nM, 7.8 nM, 2.0 nM, 0.5 nM, 0.1 nM, and 30.5 pM,) and eTox Green (final concentration 250 nM; Agilent, part number 8711008) were added. Cytotoxicity was then monitored continuously for an additional three days.

5-FU dose-dependent effect on cell cycle progression of HT-1080 cells

HT-1080 polyclonal FUCCI cells (provided by Vigen Biotechnology, Zhenjiang, China) were produced by transducing the parental cells with FUCCI lentiviruses expressing mKate2-hCdt1 (red) and mAG-hGeminin (green). These genes were integrated into a multifunctional lentiviral vector to achieve simultaneous expression in one cell, and the transduced cells

were selected with Blasticidin S. To generate HT-1080 FUCCI cells with stable FUCCI expression and low heterogeneity, we screened monoclonal cell lines using the limiting dilution method and the xCELLigence RTCA eSight system (Please see the published application note Limiting Dilution Method Using the Agilent xCELLigence RTCA eSight System for details). The HT-1080 FUCCI cells were then seeded into a 96-well plate at 5K cells/well and treated with different concentrations of 5-FU. The cell cycle was monitored continuously for 48 hours.

All of the described assays were performed on cradles four or five of the RTCA eSight (which collect images only), with four fields of view (FOVs) captured every one or two hours using the 10x objective. Note that the Cell-by-Cell Analysis function on the Schedule page of the RTCA eSight software is enabled by default for regular assays with 96-well plates. Uncheck this function to save data acquisition and image processing time if cell-by-cell analysis is not required. While brightfield settings were automatically
adjusted by the instrument, exposures in the red and green channels were manually set to 100—400 ms (depending on the brightness of the fluorescence). To minimize the potential phototoxicity of blue light, it is recommended to set the exposure time of the blue channel to within 100 ms.

Results and discussion

Quantification of suspension and adherent cell proliferation using live-cell imaging

To validate the accuracy of the brightfield segmentation, a K562 suspension cell and a A549 adherent cell proliferation assay were conducted. Figure 2A shows images of K562 cells with a seeding density of 10K cells/well at different times

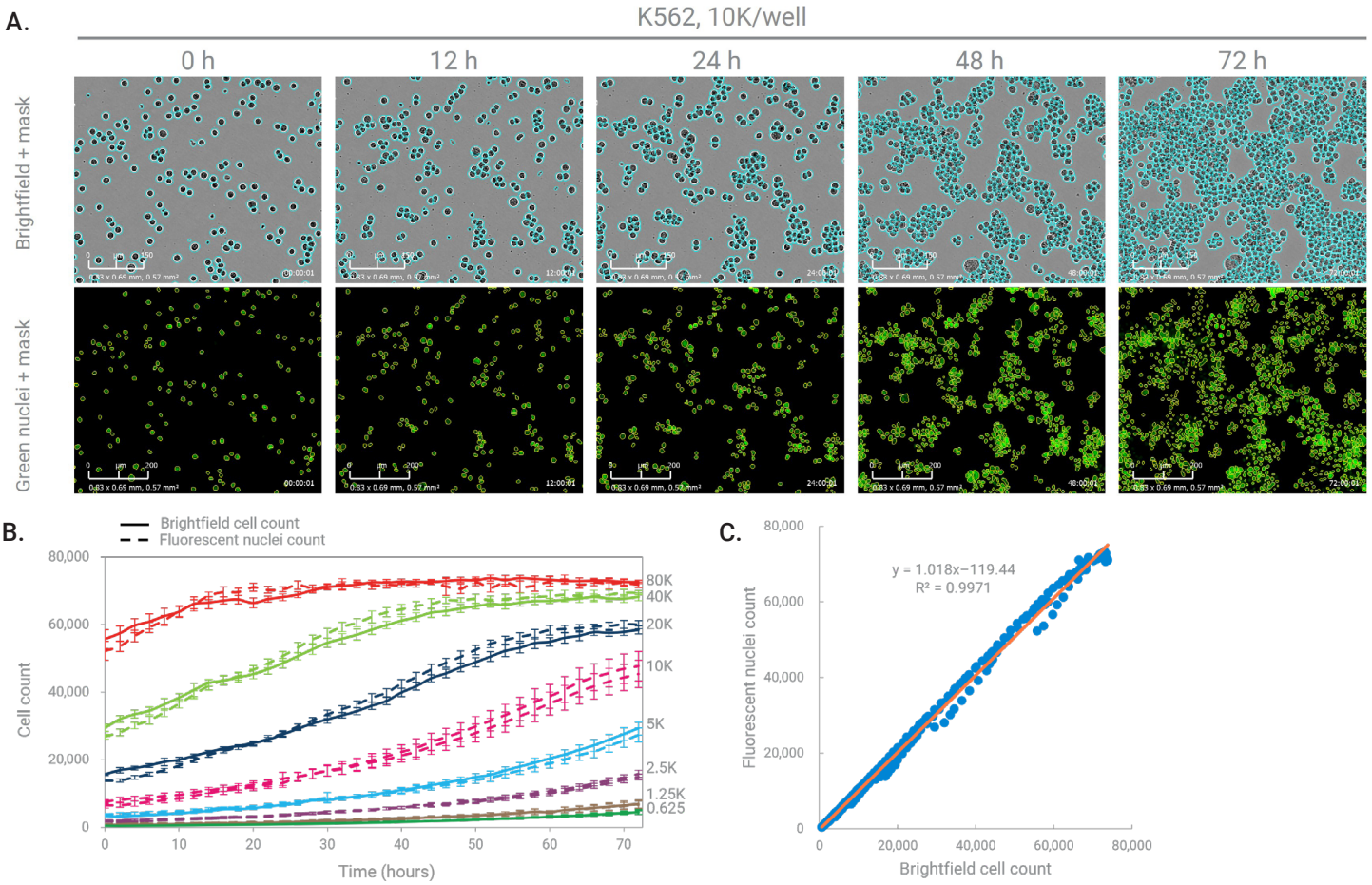

Figure 2. Accurate suspension cell counting based on brightfield. K562 Green cells generated by transducing with eLenti Green were seeded into a 96-well plate at a series of densities. Four fields of each well were captured in brightfield and green channels once per two hours over three days using the 10x objective. (A) Selected images show single-cell segmentation of K562 cells based on brightfield with an initial seeded number of 10K cells/well at different times. The first row shows the images of brightfield and brightfield mask (cyan); the second row shows the images of green nuclei and its mask in yellow. (B) The brightfield label-free cell count (solid line) and green fluorescence nuclei count (dashed line) are plotted as a function of time (N = 4). (C) The correlation of cell count data over 72 hours has an R^2 value of 0.997 with a slope of 1.018. The values shown are the mean of four replicates.

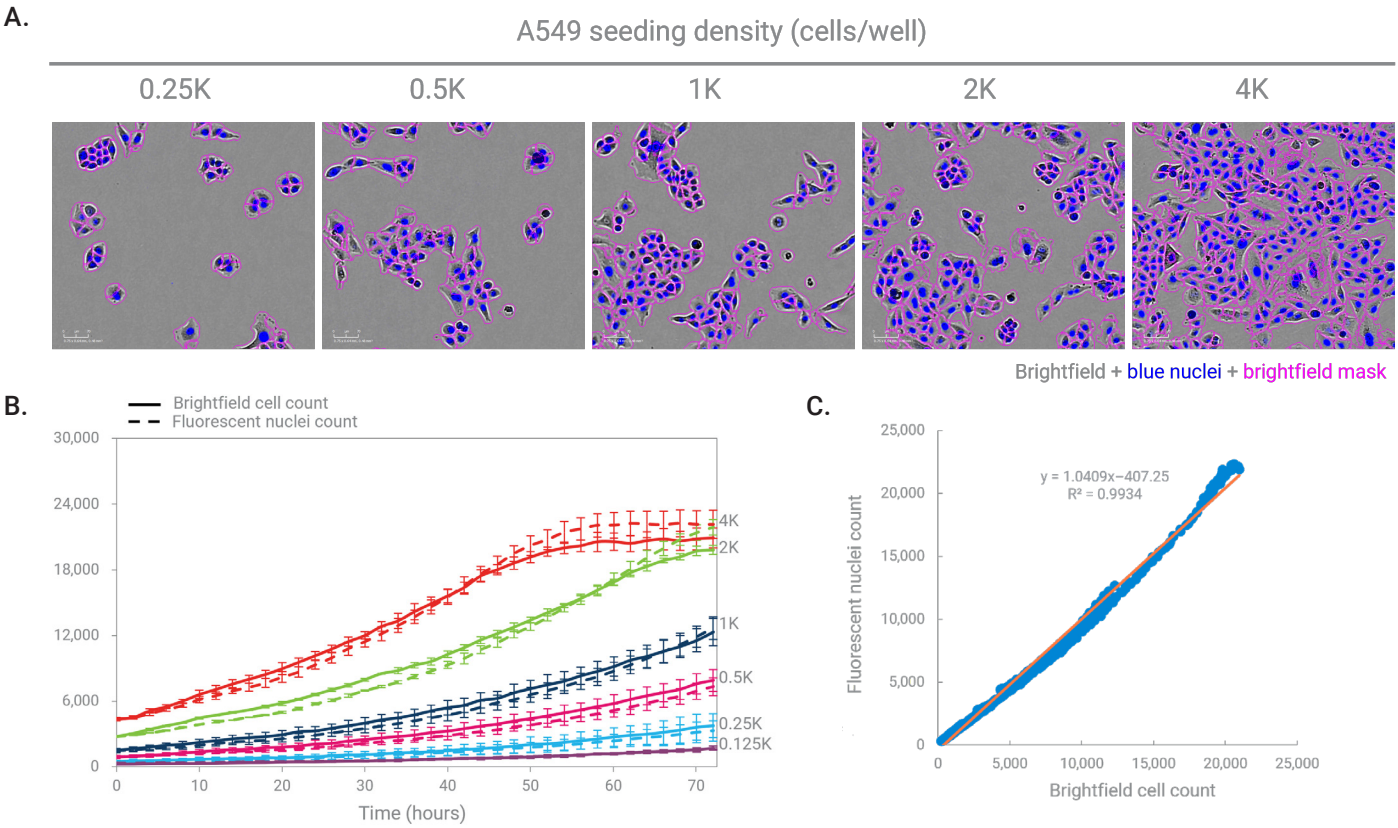

Figure 3. Accurate adherent cell counting based on brightfield imaging. A549 Blue cells were generated by transducing with eLenti Blue and were seeded into a 96-well plate at a series of seeding densities. Four fields of view were captured for each well in brightfield and green channels once per two hours over three days using the 10x objective. (A) Selected images show single-cell segmentation of A549 cells based on brightfield with a series of densities at 48 hours after seeding. (B) The brightfield label-free cell count (solid line) and fluorescent nuclei count (dashed line) are plotted as a function of time (N = 3). (C) The correlation of count data over 72 hours has an R^2 value of 0.993 with a slope of 1.041. The values shown are the mean of three replicates.

after seeding. In Figure 3A, images of A549 cells at various seeding densities 48 hours after seeding are shown. The outlines of the cell segmentation mask strongly agree with the cell borders (based on grayscale images), particularly for adherent cells. The robustness of the segmentation algorithm was also confirmed by the fact that the cell proliferation curves based on label-free cell counts and fluorescent nuclear counts strongly match up (Figures 2B and 3B). A correlation (R^2) value of 0.997 with a slope of 1.018 between the label-free cell counts and fluorescent nuclear counts in suspension K562 cells over 72 hours is observed in Figure 2C (all data in Figure 2B was included). An R^2 value of 0.993 with a slope of 1.041 was obtained in adherent A549 cells as shown in Figure 3C.

HER2 expression characterization by anti-HER2 BV421 antibody labeling

HER2 is a proto-oncogene that causes division and proliferation of cancer cells and is abnormally expressed in cancers such as breast, ovarian, and lung cancers, making it an important target for tumor-targeted therapeutic agents.[5,6]

Here, we applied our cell-by-cell analysis technique to characterize the HER2 expression of different cell lines, including lung and breast cancer cells, A549 and HCC1954, respectively. The IgG isotype antibody or anti-HER2 BV421 antibody was added to wells correspondingly at 24 hours post-cell-seeding. Both images and histograms show a lack of HER2 expression in A549 cells and high HER2 expression in HCC1954 cells (Figure 4), consistent with existing literature.

Camptothecin-induced cytotoxicity on HT-1080 cells

In addition, we investigated the response of HT-1080 Red cells to CPT, a cytotoxic alkaloid and inhibitor of DNA topoisomerase 1, which blocks DNA synthesis and has potent antitumor activity. In this study, 50 µL of medium containing both CPT and eTox Green (which selectively stains the nuclei of dead cells) was added at 24 hours post-cell-seeding, and the plate was monitored continuously in the RTCA eSight system. The Cell-by-Cell Analysis module enabled classification of cells into different cytotoxic stages based

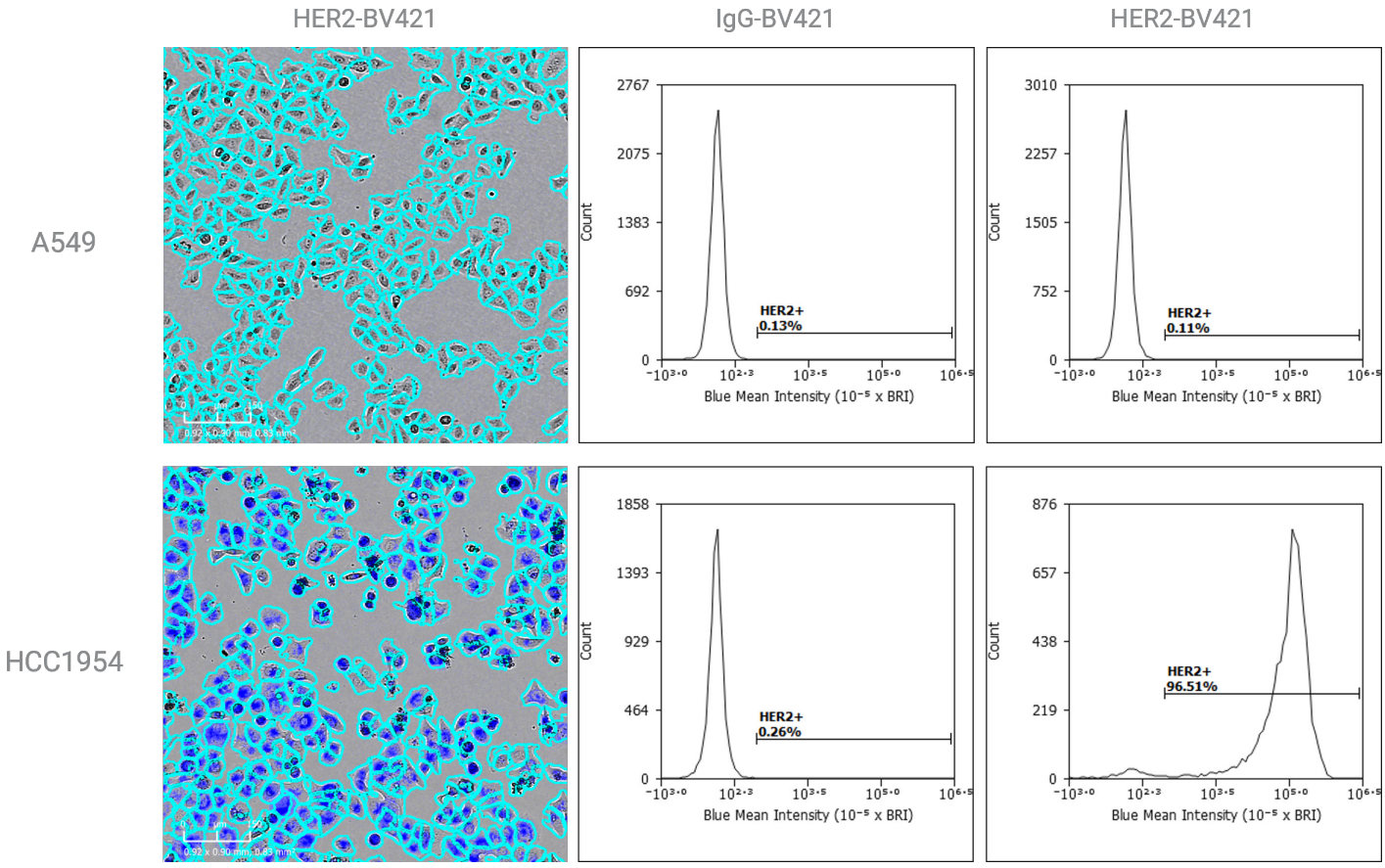

Figure 4. A549 or HCC1954 cells were characterized for HER2 expression. Images of cells labeled by the anti-HER2 BV421 antibody and fluorescence histograms of cells with IgG isotype antibody or HER2 antibody labeling from Agilent RTCA eSight show a lack of HER2 expression in A549 cells and homogenous high expression in HCC1954 cells.

on the fluorescence intensity of fluorescent protein in nuclei (eLenti Red, Red) and DNA staining of dead cells (eTox Green, Green) within the segmentation range of individual cells.

From the red/green density plot, the cells are classified into nuclei-red-positive (live cell), eTox-Green-positive (dead cell), double-positive (dying cell), and double-negative (debris) populations using a quadrant gate (Figures 5B). After treating cells with 2 µM CPT for 24 hours, the frequencies of nuclear-red-positive, eTox-Green-positive, and double-positive populations are 52.31%, 11.60%, and 25.8%, respectively (Figure 5B, upper). This was compared to 91.92%, 0.013%, and 3.57% in the vehicle control, respectively (Figure 5B, lower), suggesting a cytotoxicity effect of CPT.

Different populations in the density plot are also indicated in the cell images by the masks with the same color as the gate (Figure 5A). Cell subpopulation frequencies change throughout the time course of the experiment as a function of CPT treatment (Figure 5C). After CPT treatment, there is a significant decrease in the nuclear-red population, indicating loss of viable cells, and an increase in both the double-positive population and green-positive population, indicating cell death. In the vehicle control, the population distribution is stable. Finally, the frequencies of nuclear-red cells and eTox-Green cells with different concentrations of CPT treatment were plotted over time (Figures 5D and 5E). The half maximal inhibitory concentration (IC_{50}) values calculated from live cell and dead cell frequencies are 330.85 and 233.50 nM, respectively.

5-FU dose-dependent effect on cell cycle progression of HT-1080 cells

FUCCI is a set of fluorescent probes that enable the visualization of cell cycle progression in living cells. Cdt1, which accumulates only in the G1 phase of the cell cycle, is fused with a red fluorescent protein (mKate2), while Geminin, which accumulates in the S/G2/M phases, is fused with a green fluorescent protein (mAG1). The fluorescent intensity

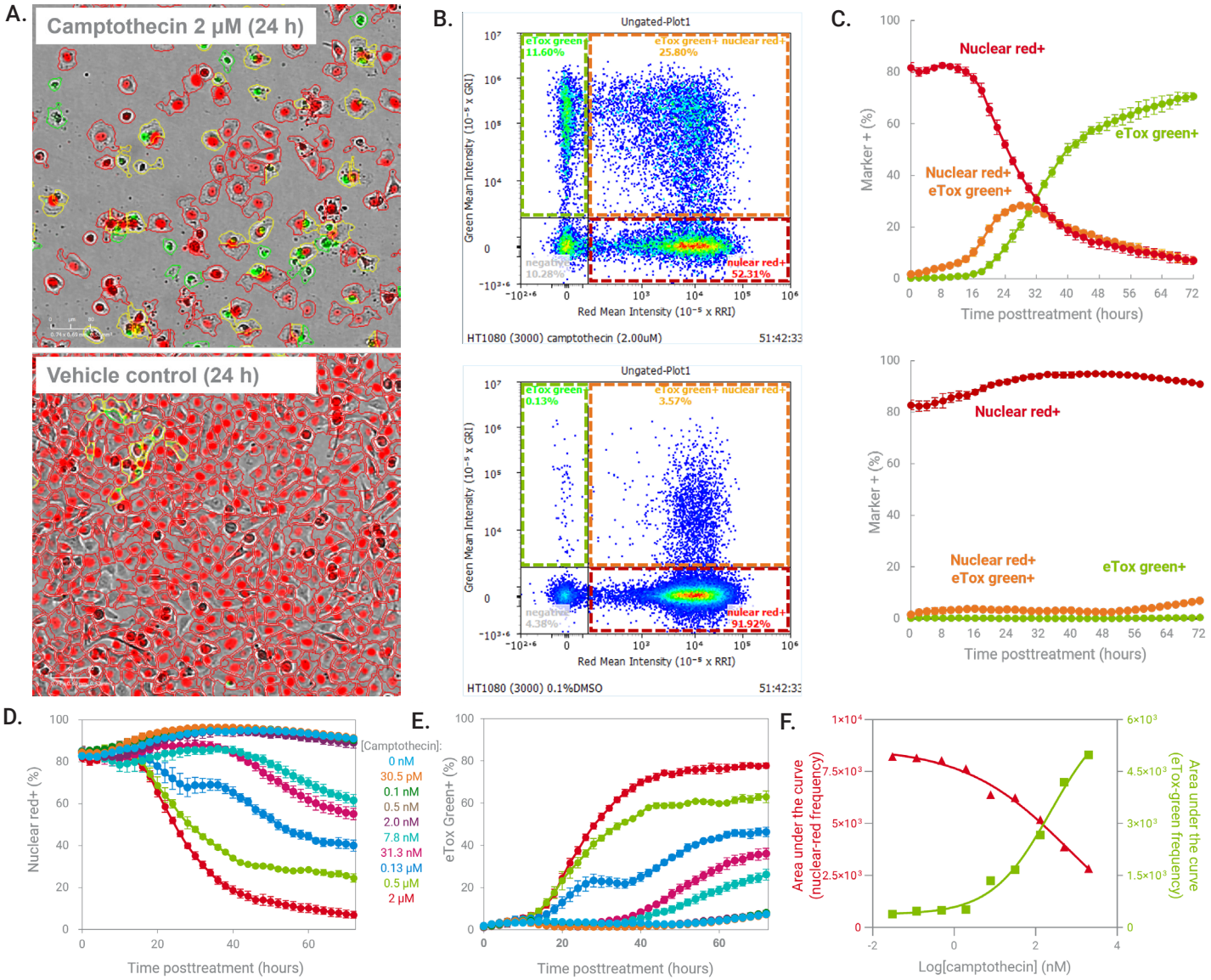

Figure 5. Cytotoxic effects of camptothecin (CPT) on HT-1080 cells. HT-1080 cells expressing red fluorescent protein in the nuclei were produced through transduction with Agilent eLenti Red. One day post-cell-seeding, cells were treated with CPT and eTox Green reagent to selectively stain nuclei of dead cells. (A) Representative images 24 hours posttreatment; mask outlines denote cell classification (Red = RFP + eTox –, Orange = RFP + eTox +, Green = GFP – eTox +). (B) Red/green density plots 24 hours posttreatment classify cell subsets (upper: CPT treatment; lower: vehicle treatment) using Agilent RTCA eSight Cell-by-Cell Analysis. (C) Frequency changes of cell subpopulations in (B) throughout the experiment timeline (upper: CPT; lower: vehicle). (D) Time-course frequency plot of "nuclear RFP+" cells at various CPT concentrations. (E) Time-course frequency plot of "nuclear eTox Green+" cells at various CPT concentrations. (F) Dose-response curves showing the area under the curves (AUC) from (D) and (E).

of each color is dependent on the expression level of the Cdt1 and Geminin proteins. Therefore, cells fluoresce green during S/G2/M and red during G1. Cells are colorless during the transition from M to G1 and yellow in the transition from G1 to S phase. The cells show reciprocally oscillating signals of the two fluorescent proteins, mAG1 and mKate2, depending on their cell cycle phase.

The RTCA eSight system can automatically acquire and analyze images of FUCCI-expressing cells in real time to identify the cell cycle phase of individual cells and thereby measure cell cycle dynamics. The schematic in Figure 6A shows the expression of green and red fluorescent proteins throughout cell cycle progression, and sequential images in Figure 6B show cycling cells over time. The two cells with red

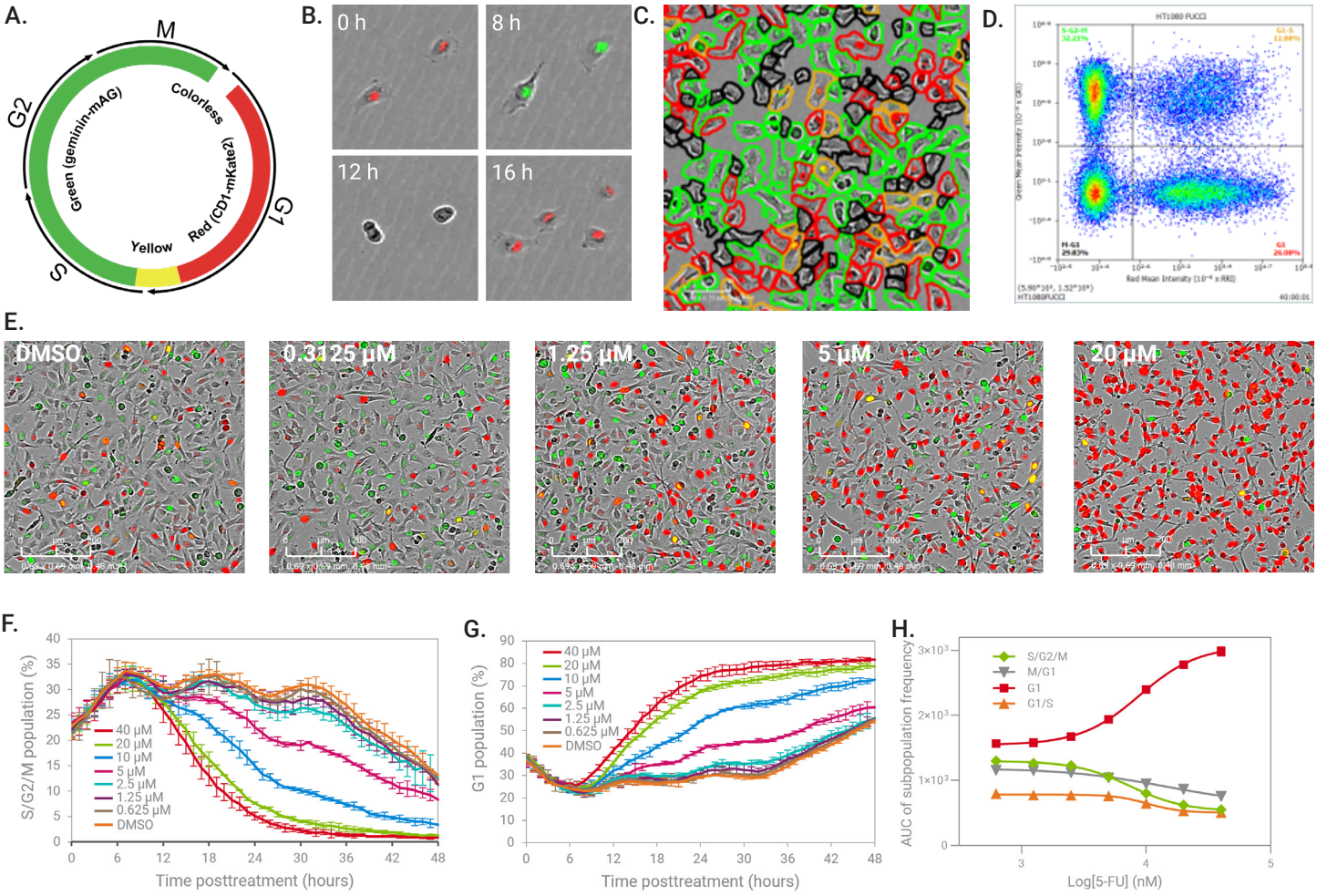

Figure 6. 5-FU dose-dependent effect on cell cycle progression of FUCCI-expressing HT-1080 cells. (A) A schematic diagram showing fluorescence changes in cell cycle progression using the FUCCI system. (B) Time-lapse imaging of FUCCI fluorescence during cell cycle progression. (C) A representative image of HT-1080 cells expressing the FUCCI system with the associated classification mask (Red = G1 phase, Orange = G1/S, Green = S/G2/M phase, Black = M/G1) is shown. (D) Cell subsets classified based on red and green fluorescence using the Agilent RTCA eSight Cell-by-Cell Analysis function. (E) Selected images at 30 hours after treatment show that 5-FU arrests the cell cycle in the G1 phase in a dose-dependent manner. (F) Concentration-response time courses of the S/G2/M population (percentage of total cells exhibiting green fluorescence). (G) Concentration-response time courses of the G1 population (percentage of total cells exhibiting red fluorescence). (H) Concentration-response curves indicate the efficacy of 5-FU on cell cycle arrest over the concentration ranges tested.

fluorescence were in G1 phase at zero hours. By eight hours, the cells had already transitioned from G1 to S/G2/M phase with green fluorescence. At 12 hours, the colorless cells were transiting from M to G1, and by 16 hours daughter cells in G1 were observed (Figure 6B). In Figure 6C, a representative image of HT-1080 cells expressing the FUCCI system is shown. Cell edges are clearly identified, as single cells are recognized based on brightfield imaging. A red and green density plot (Figure 6D) is generated by the RTCA eSight software based on red and green fluorescence intensity of each single cell area in the image. In this plot, cells can be classified into different phases by a quadrant gate. The color of the mask outlines in Figure 6C, which is the same as the color of the gates in Figure 6D (Red = G1 phase, Orange = G1 to S transition, Green = S/G2/M phase, Black = M to G1 transition), is an indication of cell cycle phase. Figure 6E shows images of cells treated with different concentrations of 5-FU. As shown, the percentage of red cells in G1 phase increases in a 5-FU dose-dependent manner, while the percentage of green cells in S/G2/M phase decreases in a 5-FU dose-dependent manner. The plots of S/G2/M percentage and G1 percentage over time in Figures 6F and 6G and concentration-response curves in Figure 6H reflect the same finding that 5-FU arrests the cell cycle in G1 phase.

Conclusion

Here, we demonstrate the utility of the Agilent xCELLigence RTCA eSight Cell-by-Cell Analysis module for cell proliferation assays, HER2 expression and characterization, camptothecin (CPT)-induced HT-1080 cell cytotoxicity assay, and 5-FU-induced cell cycle arrest studies. In summary, the Cell-by-Cell Analysis module can accurately segment suspension and adherent cells based on brightfield imaging. Additionally, the RTCA eSight enables simple, effective classification and quantification of heterogeneous populations utilizing fluorescent labeling, combined with cell viability and activity-based readouts, to ultimately define key physical characteristics and dynamic changes over time. Thereby, live-cell imaging analysis supported by the RTCA eSight platform spans from average analysis of the whole cell population to separated heterogeneous population analysis, greatly improving the versatility and power of the RTCA eSight live-cell imaging system.

References

1. Carter, B.; Zhao, K. The Epigenetic Basis of Cellular Heterogeneity. *Nat Rev Genet*. **2021**, *22*(4), 235–250. DOI: 10.1038/s41576-020-00300-0.
2. Alizadeh, A. A.; Aranda, V.; Bardelli, A.; Blanpain, C.; Bock, C.; Borowski, C.; Caldas, C.; Califano, A.; Doherty, M.; Elsner, M.; Esteller, M.; et al. Toward Understanding and Exploiting Tumor Heterogeneity. *Nat Med.* **2015**, *21*(8), 846–853. DOI: 10.1038/nm.3915.
3. Turnquist, C.; Watson R.A.; Protheroe, A.; Verrill, C.; Sivakumar, S. Tumor Heterogeneity: Does it Matter? *Expert Rev Anticancer Ther*. **2019**, *19*(10), 857–867 DOI: 10.1080/14737140.2019.1667236.
4. Ford, L .B.; Hansell, C. A.; Nibbs, R. J. Using Fluorescent Chemokine Uptake to Detect Chemokine Receptors by Fluorescent Activated Cell Sorting. *Methods Mol Biol.* **2013**,*1013*, 203—214 DOI: 10.1007/978-1-62703-426-5_13.
5. Guan, S. S.; Chang, J.; Cheng, C. C.; Luo, T. Y.; Ho, A. S.; Wang, C. C.; Wu, C. T.; Liu, S. H. Afatinib and its Encapsulated Polymeric Micelles Inhibits HER2-Overexpressed Colorectal Tumor Cell Growth In Vitro and In Vivo. *Oncotarget*. **2014**, *5*(13), 4868—4880. DOI: 10.18632/oncotarget.2050.
6. Noblejas-López, M. D. M.; Gandullo-Sánchez, L.; Galán-Moya, E. M.; López-Rosa, R.; Tébar-García, D.; Nieto-Jiménez, C.; Gómez-Juárez, M.; Burgos, M.; Pandiella, A.; Ocaña, A. Antitumoral Activity of a CDK9 PROTAC Compound in HER2-Positive Breast Cancer. *Int J Mol Sci*. **2022**, *23*(10), 5476. DOI:10.3390/ijms23105476.

www.agilent.com/lifesciences/esight

Published in the USA, March 4, 2025
5994-8047EN

Agilent
Trusted Answers